# THE RELATION BETWEEN INTENSITY OF INDUCTOR AND TYPE OF CELLULAR DIFFERENTIATION OF RANA PIPIENS PRESUMPTIVE EPIDERMIS[1, 2]

LESTER G. BARTH AND LUCENA J. BARTH

*Marine Biological Laboratory, Woods Hole, Mass. and the Departments of Zoology of Columbia University and Barnard College, New York 27, N. Y.*

In a recent paper (Barth and Barth, 1962), we suggested that lithium chloride applied in various concentrations might be expected to induce the presumptive epidermis to differentiate into various cell types. Relatively low concentrations of lithium chloride regularly induced nerve, higher concentrations, pigment cells and, occasionally, very high concentrations for long periods induced muscle cells.

The present investigations are concerned with a more detailed study of the effects of lithium chloride upon the presumptive epidermis of *Rana pipiens* gastrula.

## Experimental Procedure

An important departure from the methods used in previous experiments is the omission of agar as a substrate during the period of reaggregation of the partially dissociated cells of the presumptive epidermis. The aggregates are now prepared on the glass surface of a medium-sized stender dish and transferred at desired intervals to the small-sized stender dishes used as culture dishes. The use of a glass surface rather than agar became desirable when it was found that agar has a neuralizing effect on presumptive epidermis. This effect was first found in experiments which called for a short period during which the aggregates remained on agar before being transferred to the glass-bottom culture dishes. If the contact between the aggregate and agar was less than 30 minutes, the aggregates differentiated into a sheet of epithelium with ciliated patches instead of radial nerve with oriented cells. Aggregates exposed to agar from 45 minutes to 21 hours regularly differentiated with some type of nerve cells present.

The evidence for the neuralizing effect of agar is presented in Tables I and II. The first experiment compares the cellular differentiation obtained after the aggregates have been exposed to glass, to agar, and to glass surrounded by agar for 5 hours. The "glass surrounded by agar" was achieved by covering the bottom of the dish with agar in the usual manner and then removing strips of agar and placing the aggregates on the glass surface so exposed.

When no agar is present in the dish the aggregates upon transfer to culture dishes spread out into a thin sheet of epithelium with ciliated patches. Only one aggregate formed a few nerve fibers. In contrast the aggregates which had been exposed to agar, with or without direct contact, differentiated into spreading

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TABLE I

*The neuralizing effect of agar upon presumptive epidermis*

Stage 11 gastrulae. Operating and culture medium: solution with 1 mg./ml. globulin (Bios). Agar: washed repeatedly in distilled water over prolonged period at 3–5° C. Dissolved in standard solution. Conditions: operation, dissociation in Versene and preparation of aggregates carried out on agar or glass as indicated and aggregates left for period indicated before transfer to small culture dishes without agar.

| Exp. No. | Conditions | Time, hrs. | No. of aggregates | Cellular differentiation |
|---|---|---|---|---|
| 1 | Glass surface | 5 | 30 | Epithelium |
| | Agar surface | 5 | 30 | Spreading nerve |
| | Glass surface, agar sides | 5 | 45 | Spreading nerve |
| 2 | Agar film | 7 | 40 | Epithelium |
| | | 23 | 35 | Epithelium |
| 3 | Agar, pH 6.9 | 6–8 | 75 | Radial nerve |
| | Agar, pH 8.3 | 6–8 | 75 | Radial nerve |
| | Agar, pH 7.0 | 0.5 | 35 | Epithelium |
| | Agar, pH 7.0 | 2.5 | 40 | Radial nerve |
| | Agar, pH 7.0 | 6.0 | 35 | Radial nerve |

nerve which is characterized by a network of nerve fibers and very few epithelial cells.

The neuralizing effect of agar is thus not the result of the type of surface provided by agar but is probably brought about by a diffusible substance present in the agar. Repeated washing with cold distilled water over prolonged periods

TABLE II

*Effect of nitrogen and oxygen on the neuralizing action of agar*

Time: time in hours of exposure to agar and gas

| Time, hrs. | $N_2$ | | $O_2$ | | Air | |
|---|---|---|---|---|---|---|
| | No. | Diff. | No. | Diff. | No. | Diff. |
| 0.1 | 40 | Epithelium | 30 | Ciliated masses | 40 | Epithelium |
| 0.5 | 85 | Epithelium | 30 | Epithelium | 40 | Epithelium |
| 0.75 | 35 | Epithelium | 50 | Short nerve | 40 | Short nerve |
| 1.0 | 50 | Epithelium | 40 | Short nerve | 35 | Short nerve |
| 1.5 | 115 | Epithelium | 75 | Radial nerve | 35 | Radial nerve |
| 1.75 | | | | | 40 | Radial nerve |
| 2.25 | | | | | 35 | Short nerve |
| 3.0 | 45 | Epithelium<br>Radial nerve | | | 40 | Short nerve |
| 3.3 | 35 | Radial nerve | | | | |
| 4.0 | | | 35 | Radial nerve | | |
| 5.0 | | | 15 | Radial nerve | 40 | Spreading nerve |
| 6.0 | | | | | 35 | Radial nerve |
| 7.0 | | | | | 40 | Spreading nerve |
| 7.75 | 75 | Spreading nerve<br>Radial nerve | | | 40 | Spreading nerve |
| 8.0 | 25 | Spreading nerve | 35 | Spreading nerve | | |
| 21.0 | | | | | 35 | Radial nerve |

did not remove this substance. Since streptomycin sulfate antagonizes the neural differentiation produced by agar, we tried washing the agar with this compound. Such treated agar still induced nerve, however.

The second experiment recorded in Table I deals with a special situation in which an extremely thin film of agar was obtained by pouring hot agar into a hot dish and immediately pouring out the excess. A continuous film of agar resulted upon cooling, and the aggregates exposed to such a film for 7 to 23 hours differentiated into epithelial sheets with no nerve present. We conclude that the agar surface is not the neuralizing agent.

The third experiment was prompted by the possibility that, in the preparation of the agar by boiling in our salt solution, the phosphates precipitated and the pH became elevated. Other experiments show that sodium bicarbonate added to the solution to bring the pH to 8.8 induces nerve. The data show that agar at pH 6.9, 8.3 and 7.0 induces nerve if the exposure is more than 30 minutes. Since our salt solution for culture of the cells is at pH 8.0–8.2 and does not induce nerve at these pH's, the neuralization by agar dissolved in the salt solution is not brought about by a change in pH.

Table II gives additional evidence for the neuralizing effect of agar. Aggregates are permitted to form on agar and are left there for varying lengths of time. In air and in oxygen, 45 minutes' exposure is sufficient for neuralization. Further exposure results in more and more neural differentiation with fewer and fewer epithelial cells present. No significant differences in differentiation occurred in oxygen as compared with air. In nitrogen, however, epithelial sheets with little or no nerve were obtained for as long as 90 minutes. After 3.3 hours' exposure to agar and nitrogen, differentiation was predominantly nerve in character.

As a result of the experiments recorded in Tables I and II, we decided to carry out all the operations on glass for the experiments presented in this paper. Otherwise the method is the same as outlined in Barth and Barth (1962).

## Classification of Cellular Differentiation

We will use the same designation of the types of differentiation obtained by treatment with various compounds as was used in Barth and Barth (1962). A brief description follows:

1. Epithelium: The cells spread out in a sheet of epithelial cells, patches of which become ciliated. A variant results when the aggregate fails to attach and free-swimming ciliated masses of cells result.

2. Radial nerve: Some of the cells spread out as an epithelial sheet while the cells in a centrally located mass send out nerve fibers over the surface of the epithelial sheet. Later the cells in the epithelial sheet become elongated and oriented in parallel fashion about the central mass of nerve cells. The epithelial cells do not form cilia.

3. Spreading nerve: Few epithelial cells are present and these few are scattered about the periphery of a diffuse nerve network. The latter is characterized by an extensive migration of neuroblasts to form several loci for formation of nerve fibers.

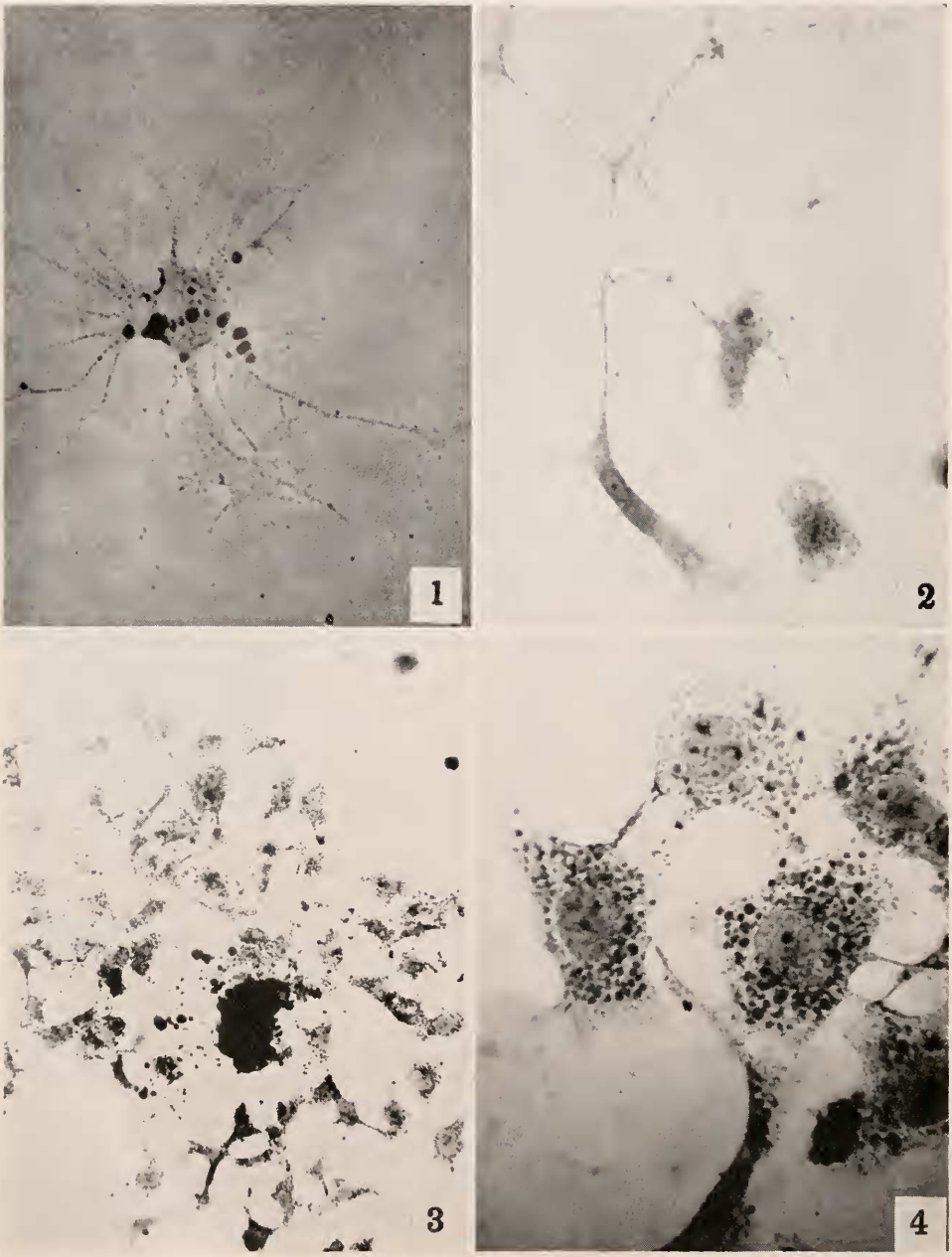


FIGURE 1. An "astrocyte" from a culture prepared by treating presumptive epidermis cells of stage 11 gastrula with a solution of lithium chloride (0.74 mg./ml. of standard solution) for 47 hours at 23° C. On return to standard solution the cells differentiated as large pigment ring cells and transformed into "astrocytes." Fixed and stained after 37 days of culture at 23°–24° C.

4. Short nerve: No epithelial cells are present. The neuroblasts remain in a dense mass and send out numerous nerve fibers which turn and run parallel to the mass of neuroblasts.

5. Pigment cells: The original aggregate spreads out and the cells appear to repel each other so that single cells, approximately equidistant from each other, result. Most of these cells develop a ring of slate-gray pigment granules in contact with the nuclear membrane. These cells later form melanophores. Some mesenchyme cells also are present.

In addition three new types of cellular differentiation have been obtained.

6. "Astrocytes": Large star-shaped cells with a large pigment ring and filaments containing granules. These cells persist for three to four weeks and resemble astrocytes. Figure 2 shows such a cell with its filaments attached to adjacent cells. Figure 1 shows a typical "astrocyte."

7. "Neuroglia": Very large cells with long thick filaments filled with granules. They first arise as large single cells and as the filaments develop they connect with the filaments of adjacent cells. The result is a network or sometimes a lattice work of cell filaments. The cells resemble neuroglia cells. Figures 3 and 4 show these cells at 4 days while Figures 5 and 6 illustrate the connections between cells on the seventh day.

8. Calcium-induced nerve: This type of cellular differentiation is not described by either radial nerve or spreading nerve or short nerve. Although there are some epithelial cells and these become oriented as in radial nerve, there are also many nerve fibers growing out from the central mass into a periphery relatively free of epithelial cells. These fibers resemble short nerve. Then, too, although there is some migration of neuroblasts as in spreading nerve, most of the neuroblasts remain in a dense central mass. For purposes of identification we will use the term calcium-induced nerve to describe the above cultures (Figs. 7 and 8).

Although any one of these types of cellular differentiation may be induced with high frequency, in any one experiment, there is almost always some other type present. For example after certain treatments with lithium chloride most of the aggregates will form pigment ring cells but a few aggregates will also form nerve cells in addition to pigment ring cells. In the tables such a result would be classified as "pigment cells."

Similarly, our controls are now epithelial sheets with ciliated patches but a few aggregates in some experiments have formed some nerve. These are still classified as "epithelium."

In addition there are some inconsistencies in the tables. These are probably the result of one or all of three variables which are not accurately controlled. First, there is the stage of the donor gastrula. While we try to use stage 11 consistently, stage 11 itself is a variable and of course the age of the presumptive epidermis is important in relation to its competence to be induced. Second, we

FIGURE 2. Same as Figure 1 and illustrates tendency of "astrocytes" to make contact with adjacent cells.

FIGURE 3. A 4-day culture of "neuroglia" cells, produced by culturing the presumptive epidermis of stage 11 gastrula in a solution containing 0.47 mg. of lithium chloride per ml. of standard solution. These cells contain no pigment rings.

FIGURE 4. Same as Figure 3 but higher magnification, showing absence of pigment rings and presence of yolk.

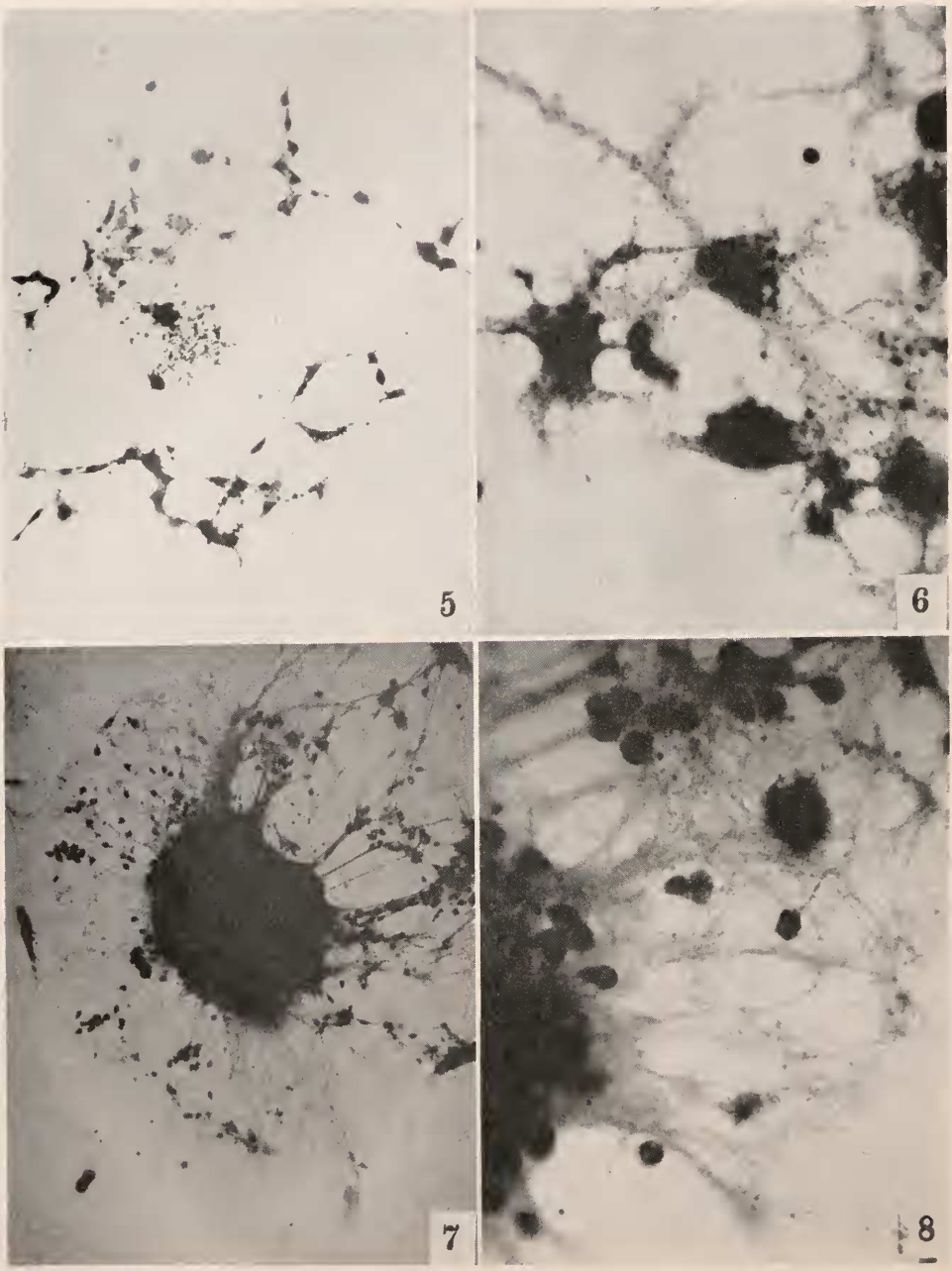


FIGURE 5. A 7-day culture of "neuroglia" cells prepared by culturing the presumptive epidermis of stage 11 gastrula in a solution containing 0.56 mg. of lithium chloride per ml. of standard solution. Most of the cells are connected with each other by protoplasmic filaments.

FIGURE 6. A detail of Figure 5, showing the granular nature of the protoplasmic filaments.

have not accurately controlled the temperature during the treatment of the aggregates. We use room temperature which is controlled by air conditioners. In early experiments we found the vibration of constant temperature incubators to have a deleterious effect on the attachment of the aggregates.

Finally the size of the aggregate we use varies and size may be important in determining the concentration of the test solution at the cell surfaces. For example, lithium chloride in low concentrations induces nerve cells while in higher concentrations pigment cells form. In a relatively large aggregate in a high concentration of lithium we find that the peripheral cells form pigment cells while the centrally located cells become nerve cells. Is this a result of the relatively large mass having been subjected to a gradient of concentrations of lithium, high at the periphery and low in the center?

## Results

We will first report the results of experiments designed to test whether inductors such as sodium bicarbonate, magnesium sulfate, ammonium sulfate and calcium chloride induce the presumptive epidermis to form various types of nerve when the neuralizing effect of agar is eliminated. Second, the results of an extensive series of experiments in which lithium chloride in various concentrations is used as an inductor for varying lengths of time will be presented.

*Sodium bicarbonate.* The addition of 1 mg./ml. of $NaHCO_3$ induces the presumptive epidermis to form nerve cells. Table III, Exp. 1, records the results of exposing the aggregates for periods varying from 0.1 hour to continuous exposure. Actually some of the aggregates have been exposed longer than the recorded time since it takes about 20 minutes to prepare them. This probably accounts for the little nerve present with a short exposure of 0.1 hour. Typically, however, a short exposure results in extensive sheets of epithelial cells, some of which are ciliated. Nine hours' treatment induces extensive radial nerve together with elongate cells oriented in parallel fashion. Some of the aggregates form short nerve with no epithelial cells present. With longer exposure or continuous exposure the aggregates exhibit no migration of epithelial cells but remain as a dense mass of cells. Nerve fibers develop slowly and turn to run parallel to the circumference of the mass of neuroblasts. Many fibers form a dense intricate pattern of fibers classified as short nerve.

Exp. 2 confirms the first experiment and was designed to test the period of competence of the presumptive epidermis for neuralization by sodium bicarbonate. Again a short exposure results in a little nerve while the control shows no nerve. The competence after 0.5 hour remains unaltered and short nerve develops in sodium bicarbonate. After 7 hours' treatment with sodium bicarbonate the aggregates form short nerve in our standard salt solution. If, however, the aggregates are prepared in a standard salt solution and remain in it for 7 hours and then are transferred to sodium bicarbonate, little or no nerve develops and the cultures resemble controls very closely. Both sets of cultures contain pre-

Figure 7. Calcium-induced nerve produced by culturing the presumptive epidermis of stage 11 gastrula in a solution containing 0.54 mg. $CaCl_2 \cdot 2H_2O$ per ml. of standard solution. Fixed at 7 days.

Figure 8. A detail of Figure 7, showing high frequency of nerve fibers.

dominantly epithelium. Thus, stage 11 presumptive epidermis loses its competence to react to added sodium bicarbonate in 7 hours at 24° C. By this time the whole gastrula controls are in stage 12.

Exp. 3 records the effects of a lower concentration of sodium bicarbonate. A sojourn of 0.5 hour in a concentration of 0.95 mg./ml. has no effect and the presumptive epidermis does not form nerve. If the aggregates remain in standard salt solution for 0.5 hour and then are cultured in 0.95 mg./ml., nerve is induced. Competence decreases after 7 hours at 19° C. and very little nerve is induced by added sodium bicarbonate.

TABLE III

*Effects of sodium bicarbonate on the differentiation of presumptive epidermal cells, stage 11*

The normal concentration of $NaHCO_3$ in our standard solution is 0.2 mg./ml. In this and the succeeding tables the table headings are to be interpreted as follows. Stage: Shumway (1940); Treatment, conc.: the final concentration of the test substance in milligrams per milliliter of standard solution; Time: the length of time in hours during which the aggregates are exposed to the test solution; Culture conc.: the concentration of the test substance in milligrams per milliliter of the standard solution to which the aggregates are transferred; No.: the number of aggregates treated; Types of cellular differentiation: as defined in text.

| Exp. No. | Treatment | | | | Culture | | | Results |
|---|---|---|---|---|---|---|---|---|
| | Conc., mg./ml. | pH | Time, hrs. | Temp., ° C. | Conc., mg./ml. | pH | No. aggregates | Types of cellular differentiation |
| 1 | 1.2 | 8.9 | 0.1 | 21 | 0.2 | 8.0 | 30 | Epithelium, little nerve |
| | 1.2 | 8.9 | 9.0 | 21 | 0.2 | 8.0 | 35 | Radial nerve, short nerve |
| | 1.2 | 8.9 | 22.0 | 21–25 | 0.2 | 8.0 | 40 | Short nerve |
| | 1.2 | 8.9 | 0.5 | 24 | 1.2 | 8.9 | 25 | Short nerve |
| 2 | 1.2 | 8.9 | 0.5 | 24 | 0.2 | 8.2 | 25 | Epithelium, little nerve |
| | 0.2 | 8.2 | 0.5 | 24 | 0.2 | 8.2 | 25 | Epithelium, no nerve |
| | 0.2 | 8.2 | 0.5 | 24 | 1.2 | 8.9 | 25 | Short nerve |
| | 1.2 | 8.9 | 7.0 | 24 | 0.2 | 8.2 | 25 | Radial nerve |
| | 0.2 | 8.2 | 7.0 | 24 | 0.2 | 8.2 | 25 | Epithelium |
| | 0.2 | 8.2 | 7.0 | 24 | 1.2 | 8.9 | 25 | Epithelium, little nerve |
| 3 | 0.95 | 8.8 | 0.5 | 19 | 0.2 | 8.1 | 35 | Epithelium, ciliated masses |
| | 0.2 | 8.1 | 0.5 | 19 | 0.95 | 8.8 | 40 | Short nerve |
| | 0.95 | 8.8 | 7.0 | 19 | 0.2 | 8.1 | 40 | Epithelium, some nerve |
| | 0.2 | 8.1 | 7.0 | 19 | 0.95 | 8.8 | 35 | Epithelium, little nerve |

*Magnesium sulfate.* Table IV summarizes the effects of various concentrations of magnesium sulfate applied for varying lengths of time. While all concentrations used induce nerve if applied long enough, the higher concentrations induce spreading nerve while the lower concentrations induce radial nerve. Similarly, if the concentration is kept constant and the time varied, long exposures result in spreading nerve while with short exposures radial nerve develops. Thus, 6.2 mg./ml. of magnesium sulfate induces some radial nerve at 1.3 hours; after 4.3 hours all radial nerve is obtained, while after 5.5 hours the cultures are mostly spreading nerve.

It is of interest to point out that the presumptive epidermis survives continuous

culture in concentrations as high as 5.2 mg./ml. This is a 25-fold increase in the concentration of magnesium sulfate used in the standard culture medium.

*Ammonium sulfate.* The effects of ammonium sulfate at two concentrations are given in Table V. At the lower concentration, 0.024 mg./ml., the presumptive epidermis survives 21 hours' exposure, but not continuous treatment. Three hours' treatment has no visible action and the presumptive epidermis spreads out as a sheet of epithelial cells. After 5 hours some radial nerve is formed,

TABLE IV

*The effects of magnesium sulfate ($MgSO_4 \cdot 7H_2O$) on the differentiation of presumptive epidermis cells (stage 11)*

| Treatment | | | Culture | | Results |
|---|---|---|---|---|---|
| Conc., mg./ml. | Time, hrs. | Temp., ° C. | Conc., mg./ml. | No. aggregates | Types of cellular differentiation |
| 2.2 | 8.3 | 24 | 0.2 | 25 | Radial nerve |
| 2.2 | 0.5 | 24 | 2.2 | 25 | Radial nerve |
| 3.2 | 4.0 | 23 | 3.2 | 75 | Radial nerve, spreading nerve |
| 4.2 | 4.0 | 23 | 4.2 | 75 | Spreading nerve, radial nerve |
| 5.2 | 0.25 | 22 | 5.2 | 75 | Spreading nerve |
| 6.2 | 1.3 | 22 | 0.2 | 25 | Epithelium, radial nerve |
| 6.2 | 4.3 | 22 | 0.2 | 25 | Radial nerve |
| 6.2 | 5.5 | 22 | 0.2 | 25 | Spreading nerve, radial nerve |
| 6.2 | 1.5 | 23 | 0.2 | 35 | Radial nerve |
| 6.2 | 4.5 | 23 | 0.2 | 40 | Radial nerve |
| 8.2 | 2.5 | 24 | 0.2 | 35 | Radial nerve |
| 8.2 | 6.0 | 24 | 0.2 | 40 | Spreading nerve |
| 8.2 | 24.0 | 23.5 | 0.2 | 35 | Spreading nerve |
| 8.2 | 28.0 | 23.5 | 0.2 | 40 | Spreading nerve |
| 10.2 | 1.3 | 22 | 0.2 | 25 | Spreading nerve, radial nerve |
| 10.2 | 4.3 | 22 | 0.2 | 25 | Spreading nerve |
| 10.2 | 5.5 | 22 | 0.2 | 25 | Spreading nerve |
| 10.2 | 1.5 | 22 | 0.2 | 40 | Radial nerve |
| 10.2 | 4.5 | 23 | 0.2 | 35 | Spreading nerve |
| 15.2 | 2.0 | 22 | 0.2 | 35 | Radial nerve |
| 15.2 | 3.0 | 22 | 0.2 | 40 | Radial nerve |

and after 8 and 21 hours' treatment all cultures exhibit radial nerve. Continuous exposure to 0.024 mg./ml. results in death.

An increase in tolerance to 0.024 mg./ml. of ammonium sulfate is shown by the results of transfers from standard solution to ammonium sulfate at various intervals. After 3 and 5 hours the presumptive epidermis begins to spread in 0.024 ammonium sulfate but soon dies, while after 8 and 21 hours epithelial sheets and ciliated masses form and persist in ammonium sulfate.

At a concentration of 0.25 mg./ml. ammonium sulfate induces radial nerve after 3 hours' exposure time, nerve after 5 hours and causes death after 7.5 hours' exposure time.

TABLE V

*The effects of ammonium sulfate on differentiation of presumptive epidermis cells (stage 11)*

| Treatment | | | Culture | | Results |
|---|---|---|---|---|---|
| Conc., mg./ml. | Time, hrs. | Temp., ° C. | Conc., mg./ml. | No. aggregates | |
| .024 | 3 | 20–21 | 0 | 25 | Epithelium |
| .024 | 5 | 20–21 | 0 | 25 | Epithelium, radial nerve |
| .024 | 8 | 20–21 | 0 | 25 | Radial nerve |
| .024 | 21 | 20–21 | 0 | 30 | Radial nerve |
| .024 | 3 | 20–21 | .024 | 25 | Dead |
| 0 | 3 | 20–21 | .024 | 25 | Epithelium → dead |
| 0 | 5 | 20–21 | .024 | 25 | Epithelium → dead |
| 0 | 8 | 20–21 | .024 | 25 | Epithelium, ciliated masses |
| 0 | 21 | 20–21 | .024 | 15 | Ciliated masses |
| .25 | 1.0 | 24.5 | 0 | 35 | Epithelium |
| .25 | 3.0 | 24.5 | 0 | 35 | Radial nerve |
| .25 | 5.0 | 24.5 | 0 | 40 | Nerve |
| .25 | 7.5 | 24.5 | 0 | 35 | Dead |

*Calcium chloride.* Various nerve patterns are obtained when calcium chloride is added to our standard salt solution. As Table VI shows, the presumptive epidermis in the standard salt solution with 0.04 mg./ml. of calcium chloride forms chiefly extensive sheets of epithelial cells with cilated patches. Only a few cells in the center of the sheet form nerve fibers. With 0.15 mg./ml. of added calcium chloride radial nerve is predominant, and when 0.25 mg./ml. is added the presumptive epidermis forms chiefly spreading nerve with few epithelial cells.

Higher concentrations, 0.54 and 1.04, induce short nerve and a new nerve pattern which has been termed "Ca-induced nerve." This latter nerve pattern has some of the characteristics of "short nerve" and some of "spreading nerve."

TABLE VI

*The effects of calcium chloride ($CaCl_2 \cdot 2H_2O$) on the differentiation of presumptive epidermis cells (stage 11)*
Standard solution contains 0.04 mg./ml.

| Treatment | | Culture | | Results |
|---|---|---|---|---|
| Conc., mg./ml. | Time, hrs. | Conc., mg./ml. | No. aggregates | |
| 0.04 | 2.0 | .04 | 75 | Epithelium, little nerve |
| 0.19 | 2.0 | .19 | 75 | Radial nerve |
| 0.29 | 2.0 | .29 | 75 | Spreading nerve, radial nerve |
| 0.54 | 7.0 | .04 | 35 | Short nerve |
| 0.54 | 2.0 | .54 | 35 | Ca-induced nerve |
| 0.54 | 1.5 | .54 | 75 | Ca-induced nerve |
| 1.04 | 7.0 | 0.04 | 40 | Short nerve |
| 1.04 | 2.0 | 1.04 | 40 | Ca-induced nerve |
| 2.54 | 3.0 | 0.04 | 35 | Radial nerve |
| 2.54 | 5.5 | 0.04 | 40 | Spreading nerve, short nerve |

There are very few epithelial cells in the periphery and many short nerve fibers but also some long nerve fibers.

As with magnesium sulfate it is interesting to note that the cells tolerate continuously a 25-fold increase in the concentration of calcium chloride.

*Lithium chloride.* The data on the effects of lithium chloride are too extensive to report in detail. Preliminary experiments indicated that the type of cellular differentiation could be controlled either by the concentration of lithium chloride or by the time of exposure. These correlations are seen in Table VII, where various concentrations have been applied to the presumptive epidermis for varying lengths of time. For example at 1.5 mg./ml. a 10-minute exposure results in epithelium with ciliated patches as in controls. As the length of treatment is increased we first obtain radial nerve, then spreading nerve and finally pigment ring cells.

TABLE VII

*The effects of lithium chloride on the differentiation of presumptive epidermis cells (stage 11)*

| Conc., mg./ml. | Duration of treatment | | | | | | |
|---|---|---|---|---|---|---|---|
| | 10 min. | 15–30 min. | 1 hr. | 2 hr. | 4 hr. | 5 hr. | 6–9 hr. |
| 0.50 | Epithelium | | Epithelium, nerve | Epithelium<br>Radial nerve<br>Pigment | | | Pigment |
| 0.75 | | | Nerve, epithelium | | Pigment, nerve<br>Epithelium | | |
| 1.00 | Epithelium | Epithelium | Radial nerve | Spreading nerve<br>Radial nerve | Pigment | Pigment | |
| 1.25 | | | Radial nerve | Spreading nerve | Pigment, nerve | Pigment | |
| 1.50 | Epithelium | Epithelium<br>Radial nerve | Radial nerve<br>Spreading nerve | Pigment<br>Spreading nerve | Pigment, nerve | Pigment | Pigment |
| 2.00 | Epithelium | | Pigment, nerve | Pigment | Pigment | Pigment | Pigment |
| 2.50 | | | | | Pigment | | |
| 3.00 | | | Pigment, nerve | Pigment | Pigment | Pigment | |
| 3.50 | | Nerve | Pigment<br>Radial nerve | | | | |
| 4.00 | | Radial nerve | Pigment, nerve | Pigment | Pigment | | |
| 6.00 | | | Pigment, nerve | Pigment, nerve | | | |

Similarly if we use one-hour exposure then with a concentration of 0.5 mg./ml. we obtain mostly epithelium with ciliated patches as in controls. Increasing the concentration to 1.0 mg./ml. results in radial nerve. With 1.5 mg./ml. we begin to find spreading nerve, and with still higher concentrations we obtain pigment ring cells.

Often two types of cellular differentiation are present in the same culture dish. This variability may be in part a result of the experimental procedure. The aggregates are prepared in lithium chloride and the time of exposure is measured from the time at which all the aggregates are prepared to the time of transfer to standard salt solution. Since it takes 20–25 minutes to prepare the 150 aggregates usually used for an experiment, some aggregates will have been exposed for as much as 25 more minutes than others. Of course the excised explant from which the aggregates are prepared has been in lithium chloride for the 20–25 minutes required for preparation of the aggregates and this fact may possibly minimize the difference in times of exposure of the aggregates.

During the course of the preceding experiments some of the aggregates were permitted to remain in lithium chloride continuously. While such treatment

usually resulted in early death of the cells, the lower concentrations, 0.56 mg./ml., sustained the cells for about three weeks. These cultures did not develop nerve nor pigment ring cells but rather contained very large scattered cells which formed thick filaments containing granules (Figs. 4, 6). The filaments of adjacent cells made contact, forming a complex lattice work or network (Figs 3, 5). These cells are tentatively identified as neuroglia cells.

Table VIII records the effects of continuous treatment of presumptive epidermis with concentrations of lithium chloride ranging from 0.10 mg./ml. to 2.00 mg./ml. The "neuroglia" type cell is obtained at concentrations of 0.56 and 0.65 mg./ml. A 7-hour exposure to these concentrations gives the expected results, namely, pigment ring cells and nerve as well as some epithelium.

TABLE VIII

*The effects of lithium chloride on the differentiation of presumptive epidermis cells (stage 11)*

| Conc. mg./ml. | Treatment | Results |
|---|---|---|
| 0.10 | continuous | Epithelium with ciliated patches; mucus cells |
| 0.20 | continuous | Ciliated masses secreting large amount of mucus; some epithelial sheets with ciliated patches |
| 0.30 | continuous | Ciliated masses and some epithelial sheets; mucus secreted |
| 0.38 | continuous | Ciliated masses with voluminous secretion of mucus containing pigment granules; astrocytes |
| 0.47 | continuous | Some ciliated masses with mucus; others form large scattered cells with protoplasmic filaments; neuroglia |
| 0.56 | continuous | All neuroglia |
| | 7 hrs. | Epithelium, scattered pigment ring cells, little nerve |
| 0.65 | continuous | All neuroglia |
| | 7 hrs. | Epithelium, pigment ring cells, nerve |
| 0.74 | continuous | Many loose cells, about 8–10 large neuroglia cells |
| 0.91 | continuous | Many loose cells, about 5–6 large neuroglia cells |
| 1.50 | continuous | Dead |
| | 4 hrs. | Pigment, nerve |
| 2.00 | continuous | Dead |
| | 4 hrs. | Pigment, nerve |

We next attempted to see if there were any other cell types between the small pigment ring cells obtained with 6–9 hours treatment with low concentrations of lithium and the "neuroglia" cells found during continuous treatment.

Table IX reveals a new cell type resembling an astrocyte. These cells arise as large scattered cells similar to "neuroglia" but possess a large ring of pigment granules near the nucleus, as do the small pigment ring cells which give rise to melanophores. The "astrocytes," however, do not give rise to melanophores, and persist as star-shaped cells for about 4 weeks (Figs. 1, 2).

The sequence of inductions following a time course is shown by the data for 0.74 mg./ml. The epithelium is first induced to become a determined nerve cell. These determined nerve cells are then induced to become determined pigment cells. Further treatment induces the determined pigment cells to become determined "astrocytes" while continuous treatment induces the determined "astrocytes" to

become "neuroglia" cells. These various cell types must be considered to be determined in the sense that placed in standard salt solution they self differentiate.

## Discussion

### *Induction by various ions*

The induction of neural cells from the presumptive epidermis by various compounds is not a new phenomenon. Many substances of widely differing chemical constitution have been shown to be neural inductors. In some instances where the substances have been implanted the action has been assigned to a toxic or sub-cytolytic action. In other cases, where the presumptive epidermis

Table IX

*The effects of lithium chloride on the differentiation of presumptive epidermis cells (stage 11)*

| Conc., mg./ml. | Time, hrs. | Results |
|---|---|---|
| 0.47 | 19.5 | Pigment cells, epithelium |
| | 25.5 | Pigment cells, epithelium |
| | continuous | Neuroglia |
| 0.56 | 5.5 | Epithelial cells, pigment cells |
| | 20.5 | Pigment cells |
| 0.74 | 6.0 | Small pigment ring → pigment cells |
| | 21.0 | Large pigment ring → astrocytes |
| 0.74 | 5.5 | Epithelium, few pigment cells, little nerve |
| | 28.0 | All pigment cells |
| | 47.0 | Large pigment ring → astrocytes |
| | continuous | Neuroglia |
| 0.91 | 5.5 | Pigment cells, nerve, epithelium |
| | 28.0 | Large pigment ring → astrocytes |
| | 47.0 | Large pigment ring → astrocytes |
| | continuous | Neuroglia |
| 0.91 | 29.0 | Pigment cells, astrocytes |
| | 46.5 | Astrocytes |
| | 56.5 | Astrocytes |
| 1.50 | 29.0 | Many dead, a few astrocytes |
| | 46.5 | Many dead, a few astrocytes |
| | 56.5 | Many dead, a few astrocytes |

is immersed in a solution of the substances, as in a conditioned medium (Niu and Twitty, 1953), it is difficult to accept a toxic action since the cells survive continuous exposure. There is of course the possibility that some cells are killed by the added substances and that the induction is actually brought about by the dead or injured cells. This latter possibility is always present since there is no way of obtaining presumptive epidermis without some injury caused by manipulation or operation. All we can do is prepare adequate controls for injury.

Our controls consisting of presumptive epidermis from the inner layer of the ectoderm of stage 11 gastrula now differentiate into epithelial sheets with ciliated patches. Only rarely does nerve appear. The injury phenomenon is definitely present and in our cultures there are always some unattached dead cells which appear about two days after the preparation of the aggregates. These dead cells

do not induce nerve, however, under the conditions of our experiment. Therefore it is probable that the induction by such ions as $Mg^{++}$, $Ca^{++}$ and $HCO_3^-$ is a result of the alteration of the living cells and not a secondary phenomenon caused by the presence of dead cells. The cells survive continuous treatment with these ions.

The significance of the experiments on induction with $Mg^{++}$, $Ca^{++}$ and $HCO_3^-$ resides in the fact that they are normally present in living inductor cells and a release of any or all of these ions during gastrulation would result in induction of a neural plate. On the other hand, the normal inductor may bring about the release of any or all of these ions within the ectoderm cells with which it comes in contact. With these widely differing interpretations of the action of $Mg^{++}$, $Ca^{++}$ and $HCO_3^-$ possible we do not feel that these studies throw any light on the naturally occurring inductors in the chorda mesoderm.

## Sequential Induction by Lithium Chloride

While the lithium ion can be definitely excluded as a natural inductor the sequence of inductions obtained by varying the concentration of lithium chloride may be of significance in the interpretation of the action of a naturally occurring inductor regardless of its chemical constitution.

Let us use epi for epidermis; $N_1$ for radial nerve; $N_2$ for spreading nerve; P for small pigment ring cells; A for "astrocytes" and Ng for "neuroglia" cells. Then we may formulate a hypothesis of 6 different pathways from presumptive epidermis to the 6 cell types:

undifferentiated ectoderm cell

| ↓ | ↓ | ↓ | ↓ | ↓ | ↓ |
|---|---|---|---|---|---|
| epi | $N_1$ | $N_2$ | P | A | NG |

The various potencies of the presumptive epidermis are shown here as diverse pathways. Lithium chloride in low concentration of 1.25 mg./ml. for one hour might be supposed to inhibit all pathways but that leading to $N_1$, *i.e.*, radial nerve. The cells are thus determined and will differentiate into nerve cells in standard solution. If, however, these determined nerve cells be exposed to lithium chloride for four hours the cells will differentiate into pigment cells (P) in standard solution. Thus, the pathway toward P was not destroyed by the one-hour treatment. Similarly, it can be shown that after P is determined, further treatment with lithium chloride results in A or Ng. Thus, lithium chloride does not appear to act by inhibiting all pathways but one, and the concept of diverse pathways is not applicable. Rather the pathway to P, for example, must pass through $N_1$ and $N_2$.

We meet with a similar inconsistency if the assumption is made that lithium chloride in low concentration stimulates the pathway leading to $N_1$ while a high concentration stimulates the pathway to P. For again we would assume that the other pathways are lost when $N_1$ is determined. But further treatment after $N_1$ is determined results in $N_2$, still further in P, and with very long times A results. Thus, the pathways do not appear to be divergent but rather they are probably arranged in some linear order.

The foregoing criticisms of the diverse-pathways concept leads to the formula-

tion of a sequence of potencies. The potencies would be present as a chain of reactions with epi and Ng at the ends of the chain. Whether we begin with epi or end with it depends upon whether we choose stimulation or inhibition as the mechanism for the induction. For example: if

$$\text{epi} \xrightarrow{1} N_1 \xrightarrow{2} N_2 \xrightarrow{3} P \xrightarrow{4} A \xrightarrow{5} \text{Ng},$$

then $Li_1$ could stimulate at 1, higher concentrations or longer times would stimulate 2, 3, 4, 5 progressively. Thus, at any time the cells might be determined to form P but the possibility of A and Ng still exists. In this formulation cells may be determined to form $N_1$ without losing the potencies in the rest of the sequence.

On the other hand if we write the sequence

$$\text{Ng} \xrightarrow{5} A \xrightarrow{4} P \xrightarrow{3} N_2 \xrightarrow{2} N_1 \xrightarrow{1} \text{epi},$$

then we express a concept of a series of reactions leading to the formation of epidermis unless stopped at some step. Mild inhibition by lithium chloride would then block the reaction 1, thus determining $N_1$, while more inhibition would block at 1 and 2, resulting in $N_2$. Still more inhibition might be expected to block 1, 2, 3, etc. Such a formulation retains all the reactions determining P when 1 and 2 are blocked, resulting in the determination of $N_2$. There would probably be some time limit on the possibility of determining P after $N_2$ is determined since the formulation shows the reactions proceeding from left to right. Presumably, with steps 1 and 2 blocked all the products of the sequence of reactions would be used in the differentiation of $N_2$. However, early in the process $N_2$ may be determined so that it will self differentiate in absence of induction but P may still be determined by a longer treatment with lithium chloride. This latter statement is simply a restatement of the results of the experiments reported in this paper.

Much of the research dealing with induction of the amphibian presumptive epidermis is difficult to interpret in light of the naturally occurring inductor or inductors. The present study is no exception. At first sight it is tempting to draw the parallel between the varying times of action of the organizer as gastrulation proceeds and the effects of lithium chloride at varying times of exposure to the presumptive epidermis. However, Mangold's transplantation of different regions of the roof of the archenteron to the blastocoele has shown that head induction and trunk induction may be obtained when the times of action are the same (Mangold, 1933). Thus, while it is definitely true that the posterior presumptive neural plate is exposed to the invaginating roof of the archenteron several hours before the anterior presumptive neural plate, this fact seems to be irrelevant to the induction process. The posterior neural plate may be induced by the posterior roof archenteron without previous contact by the anterior roof of archenteron.

It is true, however, that in the process of gastrulation the anterior roof of the archenteron first induces the presumptive spinal cord to become determined as forebrain. This determined forebrain then becomes determined as hindbrain under the influence of hindbrain inductor, and finally the determined hindbrain

is induced to become spinal cord. Thus, the preliminary induction of forebrain does not exclude the later induction of spinal cord and in this respect normal induction resembles sequential induction by lithium chloride.

It is also tempting to conclude that, since different concentrations of lithium chloride induced different cell types, the organizer is merely a graded series of concentrations of a single inductor substance. And indeed there is supporting evidence from the report by Yamada (1958) on the changes of inductive ability of the bone marrow upon heating. Possibly also the fact that denaturation of the kidney pentose nucleo protein inductor changes its inductive power from posterior neural induction to anterior neural induction might be cited (Yamada, 1958).

On the other hand, the experiments with dead roof of archenteron show no differences in inductive ability between anterior and posterior regions. If different concentrations of some inductor were responsible for anterior and posterior neural plate, one would expect these to be present in the dead cells. For the dead cells are able to induce although they lost the regional specificity for induction. The situation becomes complex indeed when we begin to suggest that the induction by dead archenteron roof is not brought about by the natural inductor.

Perhaps the fairest statement would be since lithium chloride in various concentrations induces various cell types, it makes possible an interpretation of the organizer as a concentration gradient of a single inductor, but the experiments presented in this paper do not constitute direct evidence.

## Summary

1. Presumptive epidermis of stage 11 gastrula differentiates into epithelial sheets with ciliated patches.

2. Alteration of the basic salt solution by addition of calcium chloride or magnesium sulfate or sodium bicarbonate results in the differentiation of nerve cells.

3. Calcium chloride, magnesium sulfate, sodium bicarbonate, ammonium sulfate and lithium chloride will induce nerve cells from presumptive epidermis when applied for a few hours beginning with stage 11.

4. Lithium chloride applied to the presumptive epidermis for varying lengths of time induces various cell types.

5. Lithium chloride applied in various concentrations to the presumptive epidermis induces various cell types.

6. A concept of sequential induction is introduced as a formal explanation of the lithium chloride inductions.

## LITERATURE CITED

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